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ESTERIFICATION OF STEROLS DURING DIFFERENTIATION AND CORNIFICATION OF DEVELOPING RAT EPIDERMIS*

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ABSTRACT

The proportion of sterols in esterified form increases during stratification and cornification of the fetal and newborn rat whole skin and epidermis. This change cannot be ascribed either to development of alternative biosynthetic pathways favoring formation of sterol esters or to a commensurate increase in cholesterol–fatty-acyl transferase. The activity of the latter enzyme, in fact, decreases from day 17 of gestation to 7 days postpartum. The decline in enzymatic activity is not due to the presence of inhibitors or exhaustion of endogenous substrate but may reflect changing proportions of epidermal cells in progressive stages of maturation. It is concluded that the increase in sterol esters is due to the increased availability of free fatty acids and sterols provided by the degradation of intracellular membranes during cornification.

The early observation that a greater proportion of sterols is present in ester linkage in the horny layer than in the malpighian layer suggested that sterol esterification was in some way linked to keratinization in epidermis [1, 2]. Although the original observations have been neither systematically explored nor disputed, sporadic reports have suggested that the proportion of sterol esters in both the epidermis and surface lipids [3, 4] is low in abnormal keratinization (psoriasis); the latter observation has been questioned in more recent investigations [5]. In studies of lipid metabolism of the developing epidermis of the embryonic chick, we have demonstrated an increase in the proportion of esterified sterols correlating with differentiation and cornification [6]. Moreover, increased synthesis of sterol esters as well as other neutral lipid esters, e.g., wax esters and triglycerides, along with diminished synthesis of phospholipids was observed in concert with cornification in chick epidermis developing either in the embryo or in organ culture [7]. These findings have raised once again the question as to how these changes in lipid metabolism and specifically in sterol metabolism are linked to differentiation and cornification of epidermis.

As previously demonstrated in this laboratory, sterol esterification in epidermis is mediated by a cholesterol–fatty-acyl transferase which requires only substrate fatty-acyl–Co A and free sterols [8]. The present studies were undertaken to determine whether (a) increased proportions of sterols in ester linkage are characteristic of differentiation and cornification in fetal mammalian epidermis as they are in the avian epidermis, (b) whether development of a mechanism for sterol esterification correlates with either an increasing proportion of sterol esters or cornification in fetal epidermis, or (c) whether enhanced sterol esterification during differentiation and cornification is consequent to other changes in the metabolic milieu of the epidermal cell.

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METHODS AND MATERIALS

Tissue. Pregnant rats (Sprague-Dawley) were obtained from Charles Rivers Laboratories at 15 days of gestation. Rat fetuses were removed by caesarean section from mothers anesthetized with nembutal. Fetuses and neonates were sacrificed by decapitation. Skin was removed from the dorsal surface and freed of subcutaneous tissue. The epidermis was separated from the dermis immediately after immersion in distilled water at 50° for 50 sec.

Esterification of cholesterol in vitro. Whole skin, epidermis, or dermis was homogenized in cold 0.1 M $KHPO_4$ buffer (pH 7.4) prior to use. Homogenates (1:10 or 1:20 [w/v]) of whole skin, epidermis, or dermis were centrifuged at 700 × *g* for 10 min. Supernatant fractions in amounts equivalent to 10 or 50 mg of initial tissue weight were incubated with 0.4 μmoles CoA, 4.0 μmoles ATP, and 2.0 mg albumin in a final volume of 0.8 ml. 1 μCi of cholesterol-4 ^{14}C (6–30 μg) was added in 10 μl acetone. In some experiments 0.1 μmoles sodium oleate complexed to fatty acid-poor albumin was added to the incubation mixture. Incubations were carried out for 1 hr at 37° C in a Dubnoff metabolic shaker.

For experiments with subcellular fractions of epidermis, homogenates were prepared in 0.3 M sucrose–0.1 M $KHPO_4$ buffer. 700 × *g* supernatant fractions were centrifuged in a Spinco preparative ultracentrifuge for 1 hr at 104,000 × *g* to obtain cytosol and particulate fractions.

Analyses. Concentrations of free and esterified sterols were determined in crude homogenates of whole skin, epidermis, and dermis as well as in the 700 × *g* supernatant fractions and subsequent subcellular fractions. Homogenates were extracted with chloroform–methanol by the method of Folch et al [9]. Chloroform extracts of total lipids were passed over columns of silicic acid, and sterol esters were extracted with 15% benzene–hexane followed by elution of free sterols with 20% ether–hexane. Sterols were estimated by the method of Liebermann–Burchard as modified by Martensson [10] with readings at 410 mμ at 2 and 40 min. Samples were

kept in the dark throughout and incubated at 25° C. Standards of cholesterol, cholesterol oleate, and dehydrocholesterol were employed.†

^{14}C cholesterol esters were determined in total lipid extracts of incubated homogenates made as above. Chloroform extracts were dried, resuspended in chloroform, quantitatively applied to thin-layer plates (silica gel-G),† and chromatographed successively in benzene to 19 cm and hexane–ether–acetic acid (70:30:1) to 13 cm. Carrier cholesterol oleate was used. Sterol ester bands were identified with I_2 vapors, cut out, and radioactivity was determined in scintillation counting solutions.†

Results are expressed as ng ester sterol formed per mg tissue protein. This value was derived from the counts per min of sterol esters and specific activity of the total available free sterols. Calculations entailed the assumption that added free cholesterol was interchangeable with the endogenous sterol pool.

Protein content of homogenates was estimated by the method of Lowry [11]. Histologic examination was obtained at all time points.

RESULTS

Morphologic criteria. The morphology of the developing epidermis of the rat fetus has been extensively described and our observations correspond to those of previous investigators. Prior to day 18 of gestation, there is no evidence of cornification or a granular layer with light microscopy [12]; in contrast, Bonneville [13] has demonstrated appearance of cornified cells early in gestation and has found granules resembling small keratohyalin granules as early as day 11 by electron microscopy. Days 18–19 mark the time of the appearance of the first granular layers and is followed within 1 day by the formation of a multicellular layer of stratum corneum. By day 22 (term) the granular layer is comprised of approximately 5 rows of parallel cells with increasingly large granules; the horny layer is highly developed. Postpartum, this extensive granular layer persists but begins to show some regression by day 7 as the morphology approaches that of the thin epidermis of adult skin.

For the present studies we selected days 17, 19, and 22 of gestation and days 3 and 7 postpartum in order to follow the development of the granular and horny layer and the progressive contribution by developing pilosebaceous structures.

Concentration of sterol and sterol esters. Total sterols (free + esterified) were estimated in crude whole homogenates of unincubated dorsal skin at days 17, 19, and 22 of gestation and day 7 postpartum (Table I). The lowest concentration (both in terms of wet weight and tissue protein) was observed at day 22 when the epidermis was well cornified but dermal appendages were not fully developed. Whole skin total sterols increased by day 7 postpartum; this increase was due to a rising concentration of sterols in the dermis while epidermal sterols showed no increase. We were unable to obtain adequate preparations of separated intact epidermis at days 17 and 19 to measure sterol concentrations.

The percentage of sterols in ester linkage was higher at day 19 than at day 22 in whole skin and the percentage increased again by 7 days postpartum. This increase was due to rising proportions of sterol esters in both dermis and epidermis. In epidermis the increase represented an absolute rise in the concentration of ester sterol from 4.0 μg to 6.1 μg per mg protein with a concomitant decline in free sterols.

Fast-acting versus slow-acting sterols. Because of the high levels of sterols of the $\Delta 7$ cholesterol type in rat skin, a large proportion of fast-acting sterols may be anticipated [14, 15]. To determine whether the change in sterol esters represented the emergence of an alternate pathway of biosynthesis of sterols occurring in a predominantly esterified form [14], concentrations of fast- and slow-acting sterols were estimated in both the free and esterified fractions. As shown in Table II, there was an increase in the percentage of fast-acting sterols between day 22 of gestation and 7 days postpartum in both the ester and free sterol fractions. Although fast-acting sterols were at least twice as prevalent in the ester fraction, the ratio of the micrograms of fast-acting sterols in the ester vs. the free form remained constant. It thus seems likely that pathways producing fast-acting sterols in ester form are present prior to cornification and disproportionate development cannot account for an increase in sterol esters.

Esterification of ^{14}C cholesterol. Preliminary experiments demonstrated the necessity of including estimates of the endogenous pool in assessing sterol esterification in homogenate preparations. When esterification was assessed only on the basis of the counts per min in sterol ester fractions, it was found that incorporation of ^{14}C cholesterol did not increase proportionately with tissue concentration (Table III). However, when the concentration of endogenous free sterol was taken into consideration and calculations were based upon the specific activity of the total free sterol substrate, the formation of sterol esters was shown to be proportional to the amount of added homogenate:

$$\text{S.A.} = \frac{\text{cpm } ^{14}\text{C cholesterol added}}{\text{ng endogenous + added free sterol}}$$

$$\text{ng ester formed} = \frac{\text{cpm sterol esters}}{\text{S.A.}}$$

Table IV shows the results of a single serial experiment conducted from day 17 of gestation through day 7 postpartum in whole skin, epidermis, and dermis. In this experiment each time point represents the results of 2 litters from a group of rats impregnated at the same time.

Data represent the mean results from triplicate

† Sterol standards were obtained from Applied Science Laboratories. Silica gel-G plates were obtained from Merck Company (Darmstadt, Germany). Counting solution was R.P.I. Scintillator in toluene (Research Products International Corporation, Elk Grove, Illinois).

TABLE I

Sterol content of rat skin

Day of gestation	17	19	22	Day 7 postpartum
Whole skin				
μg sterol/mg prot	83.1 ± 5.3	83.1 ± 12.4	54.3 ± 8.7	76.0 ± 28.4
% ester	8.0 ± 2.4	9.5 ± 1.2	5.6 ± 1.3	8.5 ± 1.3
Dermis				
μg sterol/mg prot			57.8 ± 5.8	84.4 ± 8.1
% ester			4.9 ± .7	6.8 ± .8
Epidermis				
μg sterol/mg prot			41.5 ± 7.5	41.2 ± 16.8
% ester			9.7 ± .8	14.9 ± .7

TABLE II

*"Fast-acting" sterol in rat skin**

Day of gestation	19 (whole skin)	22 (epidermis)	Day 7 postpartum (epidermis)
% fast-acting sterol			
ester sterol	31	34	42
free sterol	19	16	22
μg fast-acting ester sterol / μg fast-acting free sterol	.36	.33	.35

* See text for assay.

TABLE III

Effect of endogenous sterol on esterification of ^{14}C cholesterol

Protein: μg/vessel	230	460
^{14}C sterol ester: cpm/vessel	304	480
Endogenous free sterol: μg/vessel	19	38
Exogenous free sterol: μg/vessel	26	26
Total free sterol	45	64
Specific activity free sterol: cpm/ng	35	25
ng ester sterol formed/vessel	8.7	19.2

Assay as described in text. 0.1 and 0.2 ml homogenates of epidermis used in a total volume of 0.8 ml.

TABLE IV

Esterification of ^{14}C cholesterol

Day	Prepartum			Postpartum			
	17	19	22	1	3	5	7
Ester sterol formed ng/mg protein							
whole skin	35.2	32.5	13.8	16.9	20.4	45.3	51.3
epidermis	—	—	8.7	11.2	9.4	5.9	6.5
dermis	—	—	22.0	25.2	27.0	49.0	58.2

Assay performed as described in text. Homogenates (1:10; W:V) incubated with 1 μCi ^{14}C cholesterol (6.0 μg) per vessel. Each time point represents skin from 2 litters of a group of rats mated on the same date.

vessels. Each vessel contained 700 × *g* supernatant fraction equivalent to 50 mg of starting material, 1 μCi (6 μg) of ^{14}C cholesterol, plus the usual cofactors in a total volume of 0.8 ml. Sterol esterification was present in whole skin as early as day 17, prior to any visible evidence of cornification. Activity declined by day 22 and then gradually increased until day 7 postpartum. These increases reflected dermal activity which was consistently greater than that of the epidermis. Epidermal activity showed a moderate decline from the higher levels between day 22 and day 3 to day 7 postpartum.

A series of experiments using a more dilute homogenate (10–20 mg tissue) and higher concentrations of exogenous sterol (26 μg) again showed that sterol esterification in the epidermis was not increased between day 22 (28.9 ± 2.3 ester sterol/mg protein) and day 7 postpartum (20.8 ± 2.8), and in fact was significantly decreased ($p < .05$). This could not be attributed to changes in protein content which remained constant in the 700 × *g* supernatant fraction of epidermis.

Esterification in subcellular fractions. Esterification was assessed in cytosol and particulate fractions at days 17, 22, and 7 (Table V). The percentage of sterol esters remained the same in cytosol, but was higher in the particulate fraction at 7 days. As shown previously [8], the activity was found primarily in the particulate fraction, which was up to 5 times as active on a protein basis as cytosol. Activity of the cytosol may represent contamination with particulate matter.

Effect of exogenous substrate. Effects of exogenous cholesterol and fatty acid were assessed in three experiments at days 19, 22, and 7 postpartum (Table VI). A 5-fold increment in added cholesterol effected a 2-fold increase in the size of the total pool of free sterols. At this concentration there was no consistent effect on sterol esterification, suggesting the endogenous concentration is supramaximal for the reaction. Addition of 0.1 μmole of oleic acid did stimulate esterification significantly; nonetheless, formation of sterol esters was still less at 7 days postpartum than at 22 days prepartum.

Mixed incubations. The presence of increased amounts of ATPase, proteases, esterases, or inhibitors could have caused a decline in esterification in

TABLE V

*Esterification of ^{14}C cholesterol by subcellular fractions of skin homogenates**

Day of gestation	17	22	Day 7 post-partum
μgm total sterol/ml 700 × *g* supernatant fraction	170	115	110
cytosol	55	75	65
pellet	115	40	55
% ester sterol			
cytosol	10.3	10.0	9.2
pellet	8.7	8.9	17.2
ng ester sterol formed/mg prot			
cytosol	19.0	20.2	16.2
pellet	64.0	96.0	73.9

* See text for experimental details.

TABLE VI

Effect of exogenous substrate on esterification of ^{14}C cholesterol

Day of gestation	Sodium oleate	19	22	Day 7 post-partum
		(ng ester sterol formed/mg protein)		
^{14}C cholesterol				
6	−	83.6	20.8	20.1
6	+	224.0	67.4	31.9
6	−	68.0	18.6	19.0
6	+	147.2	34.6	30.2
6	−	59.0	19.5	16.6
30	−	47.0	24.2	13.2
30	+	192.0	58.7	23.2

Homogenates were incubated without (−) or with (+) 0.1 μEq Na oleate complexed to 3.0 mg fat-free albumin plus 1 μCi ^{14}C cholesterol (6 or 30 μgm) in a total volume of 0.8 ml. Controls contained albumin (3.0 mg).

vitro. This possibility was explored by mixing homogenates from 19-day and 22-day prepartum epidermis with 7-day postpartum epidermis (Table VII). Results are expressed as ng ester sterol formed per experimental vessel. The effects of mixing equal amounts of homogenates from 19 and 7 days or 22 and 7 days were additive, suggesting that the decline in activity was intrinsic to the ongoing reaction and did not represent an inhibitory effect on the in vitro system.

DISCUSSION

The data suggest that sterol esters increase in epidermis. This change is not well reflected in whole skin where both the total sterol content and the relative proportions of ester are high prior to significant epidermal cornification. Indeed the data suggest that fetal dermis (apart from the content of appendages) is rich in both free and esterified sterols. Nonetheless, sterol esters increased approximately 50 percent in epidermis between the end of gestation and 7 days postpartum.

The experiments were designed to elucidate the relation of this change to ongoing stratification and cornification. Assays of sterol esterifying activity would appear to suggest that such activity is present early in stratification and prior to development of the stratum corneum, and that these events are not dependent on the prior activation or development of the fatty-acyl–cholesterol transferase. It must be noted that the esterifying activity in whole skin does not prove that it is present in the nonkeratinized epidermis at days 17 and 19. However, the actual decline of whole skin activity by day 22 when epidermis has attained a significant volume and is undergoing precipitous keratinization suggests esterifying activity is not increasing in epidermis during this time.

Localization of the activity in the particulate fraction from the earliest point in time rules out a substantial or changing contamination of the system with the soluble lecithin–cholesterol transferase of plasma [16] and identifies the activity as the cholesterol–fatty-acyl transferase previously demonstrated in adult epidermis.

The increase in sterol esters and decline of free sterols in the progressively cornifying epidermis suggest that the metabolic events linked to cornification result in the increased utilization of the existing sterol esterifying mechanism. Failure to demonstrate an increase in activity suggests that the change was not due to increased amounts of enzyme. The emergence of a pathway of sterol biosynthesis favoring formation of esterified sterols could not be implicated.

TABLE VII

^{14}C cholesterol esterification of mixed homogenates

	Homogenate per vessel (ml)	Ester sterol formed per vessel (ng)
17-day whole skin	0.25	4.3
7-day postpartum epidermis	0.25	5.6
Combined	0.50	9.6
22-day epidermis	0.25	8.0
7-day postpartum epidermis	0.25	7.5
Combined	0.50	18.7

0.25 ml of 700 × *g* supernatant fraction from 1:10 homogenates were incubated separately and in combination with 1 μCi of ^{14}C cholesterol plus cofactors. 0.1 M $KHPO_4$ buffer was added where necessary to make a final volume of 0.8 ml. Results are expressed as ng esterified sterol formed per vessel. Calculations based on specific activity of total free sterols/vessel.

It thus seems likely, as originally proposed by Kooyman [2], that enhanced formation of sterol esters is due to the increased availability of free sterol and fatty acids. The present data support but do not document this hypothesis. Experiments with changing concentrations of exogenous substrate failed to demonstrate any limitation of free sterol in the cell-free system; it seems probable, however, that membrane-bound sterol may not be so readily available for esterification in the intact cell. The effect of added fatty acid was not much more helpful. Stimulation of sterol esterification by oleic acid seems to suggest that the endogenous pool is small and potentially rate-limiting. However, it has been demonstrated that the cholesterol–fatty-acyl transferase favors esterification of oleic acid and that this fatty acid stimulates esterification when other 16- and 18-carbon fatty acids do not [17]. The in vitro system is probably inadequate to document changes in the available pools of free sterol and fatty acids at intracellular sites in vivo. In view of the evident hydrolysis of phospholipids and the disintegration of sterol-bearing membranes during cornification, and the presence of a sterol-esterifying mechanism which cannot be demonstrated to increase, it seems most likely that increasing availability of substrate is the force driving the increasing formation of sterol esters.

The apparent decline in esterifying activity while sterol esters are increasing must also be considered. Experiments in which epidermis from 7-day neonates was mixed with the more active preparation at days 19 and 22 failed to show any inhibitory effect of the older epidermis, ruling out more active degradative processes or inhibitors of the enzyme. The decline could, however, reflect changing ratios in populations of cells. The activity is likely to be maximal in cells undergoing the most intensive degradative changes, i.e., the granular and transitional cells. At day 22, the transition of malpighian cells into granular cells is precipitous while stratum corneum is still increasing. By day 7 postpartum tne granular layer is less prominent (2–3 rows of cells as opposed to 5) and the process of massive cornification has probably slowed down. It may thus be postulated that activity of the cholesterol–fatty-acyl transferase at day 7 postpartum reflects a smaller proportion of cells undergoing cornification than at day 22, while the actual amount of sterol esters reflects the cumulative result of events throughout this period. Calculations of the formation of sterol esters based on the mean in vitro rate suggest that 4.6 μgm would be formed per mg epidermal protein between day 22 prepartum and day 7 postpartum. The observed increase in sterol esters in this interval was 2.1 μgm. The data are thus compatible with a steady rate of sterol esterification supported by endogenous substrates made available by the disintegration of intracellular organelles. The data also support the earlier hypothesis that sterol esterification in epidermis represents a salvage system whereby free sterols and fatty acids that are suddenly released may be stored in the dehydrated horny cell. In this sense, epidermis is similar to other tissues with large amounts of non-membrane-bound sterol which are preferentially stored in the esterified form. From the present data, a functional role cannot as yet be assigned to these esterified lipids in the stratum corneum.

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